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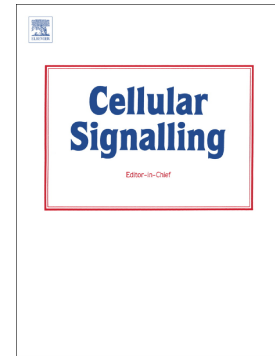
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## **A Novel Phosphorylation Site on Orexin Receptor 1 regulating OrexinA-induced GRK2-Biased Signaling**

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**Keywords:** G protein-coupled receptor; orexin receptor 1; G protein;  $\beta$ -arrestin; biased signaling; therapeutic potential

## Abstract

Drug discovery efforts targeting G protein-coupled receptors (GPCRs) have succeeded in developing multiple medications for treating various human diseases including cancer, metabolic disorders, and inflammatory disorders. These medications are broadly classified as either agonists or antagonists that respectively promote or inhibit receptor activation by endogenous stimuli. However, there has been a growing appreciation that GPCR biased signaling between G protein- and  $\beta$ -arrestin-dependent signaling in particular is a promising method for improving drug efficacy and therapy. Orexin receptor 1 (OX1R), a member of the GPCRs, is an important drug target in the central nervous system. In this study, we identified a novel regulatory phosphorylation site (Ser-262) on OX1R that abolished its capability to interact with GRK2, but did not affect its interaction with G proteins, GRK5, or  $\beta$ -arrestin1/2 activation, indicating that Ser-262 is a key amino acid for OX1R internalization that contributes to induction of GRK2-dependent biased signaling via orexin A. Our findings could potentially lead to the development of new drug targets for the prevention and treatment of insomnia, narcolepsy, and substance abuse, with fewer side effects than existing therapies.

## Abbreviations

GPCR: G-protein-coupled receptor, OX1R: Orexin receptor 1, BRET: Bioluminescence resonance energy transfer, Ser: Serine, Ala: Alanine, GRK2/5: G Protein-coupled receptor kinase 2/5, ELISA: Enzyme linked immunosorbent assay, OD: Optical density, Rluc: Renilla reniformis luciferase, EGFP: Enhanced green fluorescent protein, LC-MS/MS: Liquid chromatography coupled with tandem mass spectrometry, TIRFM: Total internal reflection fluorescence microscopy, Co-IP: Co-immunoprecipitation.

## 1. Introduction

G protein-coupled receptors (GPCRs), also known as seven-transmembrane receptors, are involved in a diverse array of physiological and pathological responses, and consequently are common targets of therapeutics [1, 2]. Binding of ligand results in activation of GPCR signaling through G proteins and  $\beta$ -arrestins [3]. In canonical G protein-dependent signaling, G proteins interact with many effector molecules to initiate signaling [4]. Furthermore, the activation of GPCRs mediated by agonist recruits and stimulates G Protein-Coupled Receptor Kinases (GRKs). Subsequently, the C-terminus or third intracellular loop (ICL3) of GPCRs is phosphorylated on the multiple serine or threonine residues, generating high-affinity arrestin-binding sites[5]. There is direct interaction between carboxyl-tail of  $\beta$ -arrestin and clathrin-adaptor protein-2 (AP-2), promoting receptor internalization and intracellular trafficking. In addition,  $\beta$ -arrestins mediate  $\beta$ -arrestin dependent signaling[6]. Signaling differences between G protein and  $\beta$ -arrestin signaling are the ultimate determinants of ligand-specific cell biological effects and thus may potentially maximize therapeutic benefits while minimize adverse effects[7]. Therefore, biased signaling between G protein and  $\beta$ -arrestin pathways is a hotspot in the field, and several biased ligands and receptors have been identified that signal preferentially, i.e., through either G protein- or  $\beta$ -arrestin-mediated pathways [8, 9]. For example, our preliminary study revealed that the 348 amino acid in carboxyl-terminal of apelin receptor (APJ), a GPCR family member, is required for biased G protein-independent APJ signaling mediated by GRKs and  $\beta$ -arrestins [10].

Orexin/hypocretin receptor 1 (OX1R) is the member of the GPCR rhodopsin family, and as such, heterotrimeric G-proteins are the main mediators of signal transduction, and  $\beta$ -arrestins are also an equally common interaction partner[11]. Many evidences show OX1R is more related to addiction, feeding, reward, emotion and motivation [12-14], for example, Gary Aston-Jones *et al.*[15] found that OX1R signaling increases motivation for cocaine-associated cues, and intra-ventral

tegmental area administration of orexin A is sufficient to reinstate previously extinguished drug-seeking behavior. Therefore, OX1R was developed as a target for novel pharmacotherapeutics, and orexin receptor-targeting molecules have been explored, in particular antagonists, such as single orexin-receptor antagonists (SORAs) and dual orexin-receptor antagonists (DORAs), to treat some diseases, such as addictive disorders and insomnia[16-18]. However, it is still unclear how OX1R mediates its G protein and  $\beta$ -dependent signaling pathways.

In this study, we monitored the ligand-induced phosphorylation sites of OX1R and performed mutagenesis to determine their roles, with the goal of precisely identifying regulatory phosphorylation sites of OX1R and exploring the effect of phosphorylation on signaling. We found that Ser-262 is a key phosphorylation site for OX1R, and after Ser-262 was replaced with Ala, orexin A fails to induce GRK2 recruitment and terminates GRK2-dependent signaling, suggesting that Ser-262 on OX1R regulating orexin A induced GRK2 biased signaling pathway.

## 2. Materials and methods

### 2.1 Plasmid Constructs and Mutagenesis

The plasmid pcDNA3.1(+)-OX1R was purchased from the Missouri S&T cDNA Resource Center.  $\beta$ -arrestin1-Rluc and  $\beta$ -arrestin2-Rluc was generated as described previously [19]. Plasmid encoding Golgi-EYFP was kindly provided by Professor Katharine Herrick-Davis (Center for Neuropharmacology & Neuroscience and Department of Psychiatry, Albany Medical College, USA). AP2u2-mCherry (AP2-mCherry) was a gift from Christien Merrifield (Addgene plasmid #27672; <http://n2t.net/addgene:27672>, RRID: Addgene\_27672). Overlap extension PCR was used to construct OX1R mutants. The high-fidelity *Pfu* polymerase was used. The primers were OX1R: 5'-CCGGAATTCATCGAGCCCTCAGCCACCCCAGG-3' (*EcoRI*) and 5'-CCGCTCGAGGGGGCAGCACTGTGGTGACGCTGGT-3' (*XhoI*), or OX1R-S262A mutagenic primers: 5'-TCTGAAGCGCCCCGCAGACCAGCTGGGG-3' and 5'-CCCCAGCTGGTCTCCCGGGCGCTTCCA-3'. After digestion with *EcoRI* and *XhoI* (Takara, Japan), the mutagenized OX1R cDNA was ligated into pcDNA3.1(+) vector. The correct constructs were verified by sequencing (Sangon Biotech, Shanghai, China).

### 2.2 Cells and Transfection

Human embryonic kidney 293T (HEK293T) cells were cultured in complete Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C, 5% CO<sub>2</sub>. Transient transfection was performed using Lipofectamine 2000 (Invitrogen, California, USA).

### 2.3 Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

After transfection with OX1R for 24h, HEK293T cells were treated with 100 nM orexin A (Phoenix Pharmaceuticals, California, USA) for 10 min at 37°C. Proteins

were extracted and immunoprecipitated with anti-OX1R antibody. The immunoprecipitated proteins were then incubated for 2 h with protein A/G PLUS–agarose beads. After washing, elution of OX1R complex was performed as previously described [10, 20]. The eluted protein samples were subjected to SDS-PAGE. The gel was stained with Coomassie at room temperature for 1 h, followed by destaining in water. The gel band of the target protein was cut out, incubated with 10 mM DTT and 30 mM iodoacetamide, and digested with trypsin overnight at 37°C. The complex was analyzed on an Eksigent Nano 1D Plus system (Thermo Fisher Scientific, California, USA). Peptides were concentrated and desalted on a C18 column (15 cm × 75 µm) and eluted on a Eksigent C18 column (350 µm × 0.5 mm) at 300 nl/min with the gradient of 5% B for 5 min, 5–40% B for 65 min, 40–80% B for 66 min, 80% B for 71 min, 80–5% B for 72 min, and 5% B for 90 min (solvent A: 0.1% formic acid (v/v); solvent B: 0.1% formic acid (v/v) and 80% CH<sub>3</sub>CN (v/v)).

For database correlation analysis, the Mascot search engine (Matrix Science, London, UK) was used to process MS/MS spectra. The phosphorylation and other biological modifications were searched. Using trypsin as the enzyme, we searched the IPI human database. We manually checked and validated all filtered MS/MS spectrums containing potentially phosphorylated peptide.

#### 2.4 Enzyme linked immunosorbent assay (ELISA)

To investigate the cell-surface expression of wild type (WT) OX1R and mutant OX1Rs, HEK293T cells were transiently transfected with HA-WT-OX1R, HA-OX1R-S262A, or HA-OX1R-S390A; the same amount of plasmid was used for each transfection. After 24 h, cells were subjected to 4% paraformaldehyde fixation at room temperature for 15 min. After blocking with 3% BSA for 1 h, primary antibody of rabbit polyclonal anti-HA (1:1000) was added and incubated at 4 °C overnight. Then, secondary antibody of goat anti-rabbit HRP–conjugated IgG (1:1000) was added for incubation at room temperature for 1 h. TMB Plus substrate was used for color development, and 0.2 M H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction. Absorbance at



450 nm was detected with a microplate reader (Bio-Rad, USA). Mock transfection was set up. The expression level was calculated as:  $[(OD_{\text{mutant}} - OD_{\text{mock}}) / (OD_{\text{wt}} - OD_{\text{mock}})] \times 100\%$ . ELISA was carried as described previously [8].

## 2.5 Bioluminescence resonance energy transfer (BRET) measurement

To monitor cell-surface expression of receptor, OX1R-Rluc, OX1R-S262A-Rluc, OX1R-S390A-Rluc, or Venus-kras (a plasma membrane-associated BRET acceptor) was transiently transfected into HEK293T cells. At 24 h post-transfection, cells were trypsinized, plated in a 96-well microplate in HEPES buffered phenol red-free medium (Invitrogen, California, USA), and incubated for 24 h. The coelenterazine h (5  $\mu\text{M}$ ; Promega, USA) was added. Tristar LB941 plate reader (Berthold Technologies, Bad Wildbad, Germany) with an enhanced green fluorescent protein (EGFP) filter (500–550 nm) and an Rluc filter (400–475 nm) was used for BRET measurements. To analyze the interactions between OX1R and intracellular signaling proteins (GRKs and  $\beta$ -arrestins), cells were transfected transiently with Venus/EGFP-tagged receptors (WT-OX1R-Venus/EGFP, OX1R-S262A-Venus/EGFP, or OX1R-S390A-Venus/EGFP) and Rluc-tagged intracellular signaling proteins (GRK2-Rluc, GRK5-Rluc,  $\beta$ -arrestin1-Rluc or  $\beta$ -arrestin2-Rluc) and stimulated with  $10^{-6}$  M orexin A, then we measured BRET signals as described above with Tristar LB941 plate reader[21, 22].

## 2.6 Total internal reflection fluorescence microscopy (TIRFM)

A commercial TIRF system (Leica Microsystems) was used. To avoid photobleaching, we restricted illumination within a single focal plane. The exposure times (three frames/s) were relatively short. The laser power was only 8% at cell surface. The evanescent field penetration depth was  $\sim 150$  nm[23].

## 2.7 Co-immunoprecipitation (Co-IP)

HEK293T cells were co-transfected with Myc-GRK2 and HA-OX1R or HA-OX1R-S262A for 48 h. Then, cells were treated with orexin A (100 nM) for 10 min, lysed and centrifuged at 16,000 g for 15 min at 4°C. For Co-IP, HA-Tag Rabbit

mAb (Sephacrose® Bead Conjugate) (Cell Signaling Technology, USA) was added for incubation with whole cell lysates at 4°C overnight. The beads were washed five times with 500 µl 1× cell lysis buffer. After elution with 20 µl 4× SDS-PAGE sample buffer, proteins were analyzed by Western blotting for anti-HA or anti-Myc immunoreactivity (Cell Signaling Technology, USA)[10].

## 2.8 Confocal laser scanning microscopy

After transfection with WT-OX1R-Venus or OX1R-S262A-Venus and mCherry-AP2 for 24 h, the cells were stimulated with orexin A ( $10^{-6}$  M) for various time intervals (0, 15, 30, and 60 min), and fixed as previously described. Laser scanning confocal microscope (oil-immersion 40× objective; TSC SP5, Leica, Germany) was used for observation.

## 2.9 Calcium fluorescence measurements

Cells expressing WT-OX1R, OX1R-S262A, or OX1R-S390A were seeded at  $4 \times 10^4$ – $5 \times 10^4$  cells/well in poly-D-lysine-coated black 96-well plates. After overnight culture, dose response curve: cells were stimulated with various concentrations of orexin A ( $10^{-11}$ – $10^{-5}$  M) at 37 °C, for 30 seconds, and calcium fluorescence was detected. The time-response, the cells were treated for 230 s with  $10^{-6}$  M orexin A at 37 °C. Then, Fluo-4/AM Calcium Assay Kit (Invitrogen, California, USA) was used for calcium signal detection. Briefly, 100 µl Fluo-4NW working solution was added. Cells were cultured for 30 min at 37°C, 5% CO<sub>2</sub>, and then for an additional 30 min at room temperature. Fluorescence was immediately measured on a Tristar LB941 plate reader. The calcium ratio was calculated using the formula:

$$\text{Calcium fluorescence ratio} = (F_0 - F_1) / F_1$$

where  $F_0$  is the fluorescence value after addition of agonist, and  $F_1$  is the fluorescence value before addition of agonist. We recorded the peak increase in fluorescence counts. The experiment was repeated three times.

## 2.10 Nuclear factor of activated T-cells (NFAT)/ serum response factor (SRF)

## luciferase reporter assays

Cells stably expressing WT-OX1R, OX1R-S262A, or OX1R-S390A were co-transfected with pNFAT-Luc or pSEF-Luc (PathDetect, Stratagene). After 24 h, cells were treated for 6 h with orexin A ( $10^{-6}$  M). Dual-Glo luciferase assay kits (Promega, USA) were used to detect relative luminescence units (RLU) for *Renilla* and firefly luciferase, as described previously [24].

## 2.11 Statistical analysis

GraphPad Prism 5.0 and SPSS 13.0 software were used to analyze results. Data were presented as means  $\pm$  SEM. Comparisons between two groups were analyzed with Student's paired t-test. Multiple group comparisons were conducted by one-way ANOVA.  $P < 0.05$  indicates statistically significant.

### 3. Results

#### 3.1 Identification of phosphorylation sites on OX1R by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

Serine/threonine in the C-terminus or ICL3 of GPCRs are required for receptor phosphorylation, internalization, and intracellular trafficking. However, the key residues of OX1R are unclear. Putative OX1R phosphorylation site Ser-390 in the C-terminal region and Ser-262 in ICL3 were identified using NetPhos 2.0 prediction software. Herein, HEK293T cells transfected with OX1R were treated with 100 nM orexin A for 10 min and then subjected to SDS-PAGE electrophoresis and Coomassie Brilliant Blue staining (Fig. 1A). To better identify the specific sites on OX1R that were phosphorylated by 100 nM orexin A stimulation, LC-MS/MS was performed. Ser-262 in the ICL3 (Fig. 1B) was identified as a phospho-acceptor site, indicating that this residue (Ser-262) is a phosphorylation site for OX1R.

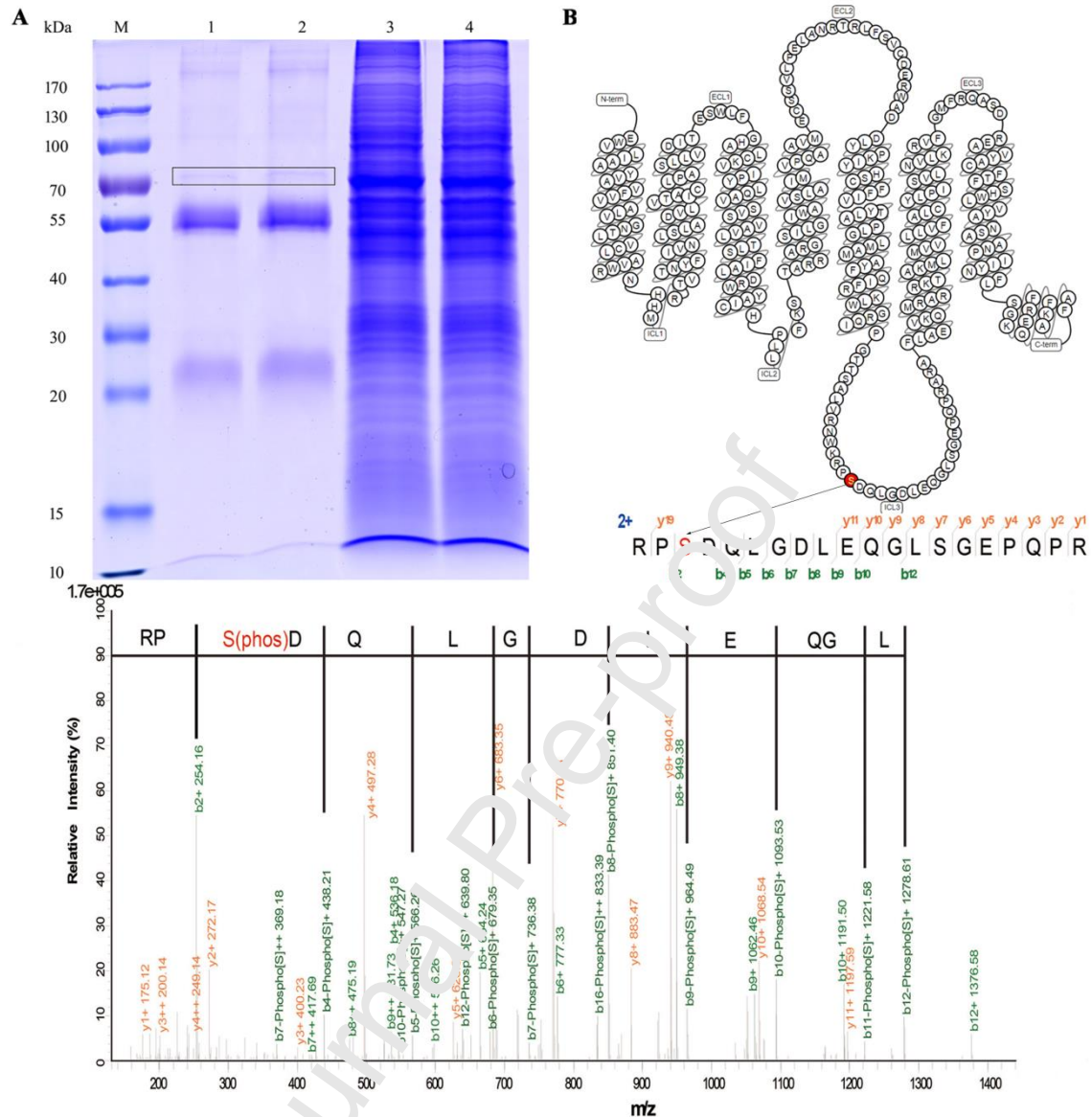


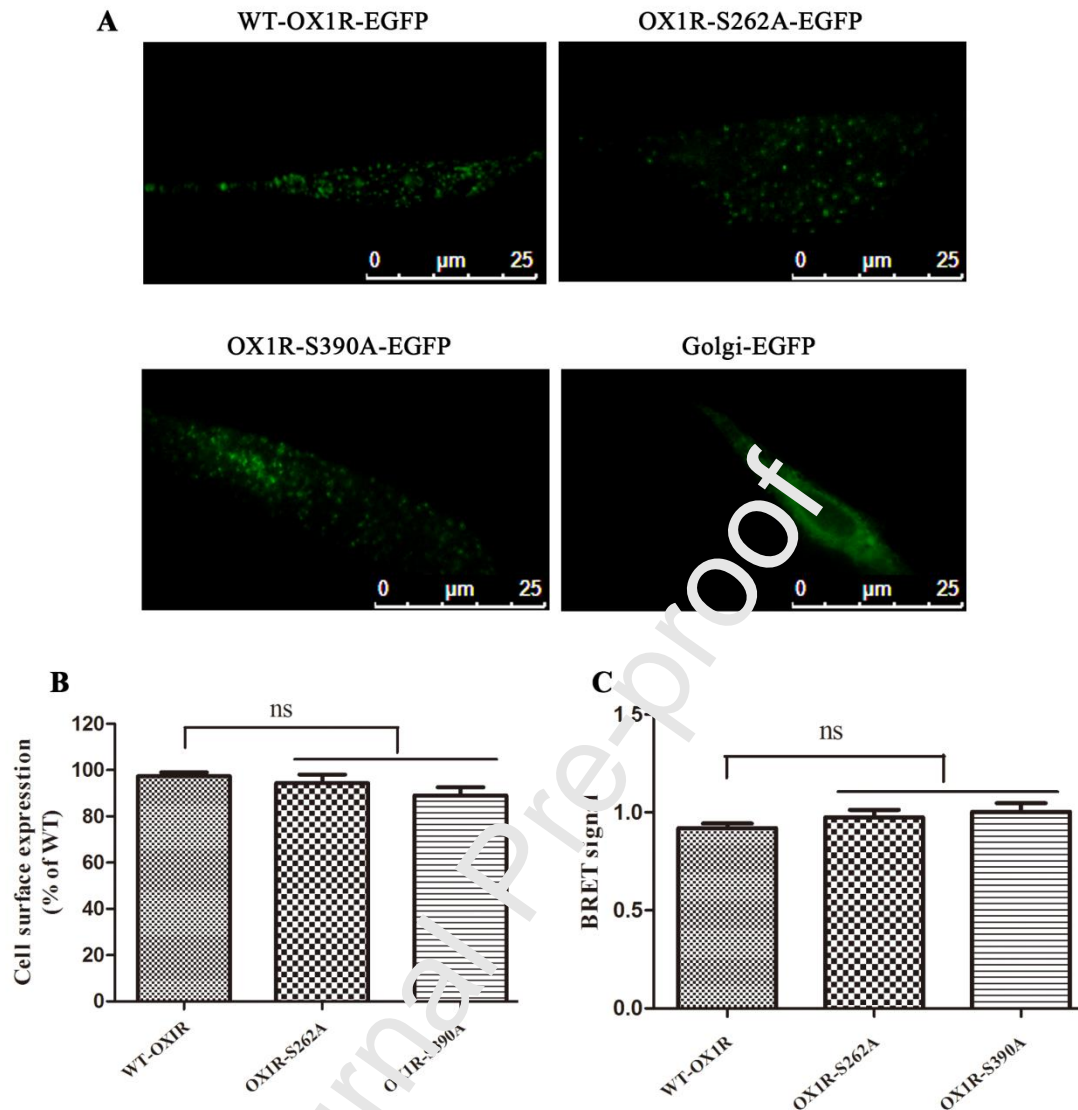
Fig 1. Identification of phosphorylation sites on OX1R by LC-MS/MS

(A) Plasmid OX1R-EGFP was transfected into HEK293T cells and treated with 100 nM orexin A for the 10 min. Proteins were resolved by SDS-PAGE and stained. Left: molecular mass markers; 1 and 2: Co-IP eluent; 3: Co-IP supernatant; 4: whole-cell protein (molecular weight of OX1R-EGFP: 75 kDa) (B) Protein was extracted and subjected to tryptic digestion, and then analyzed by LC-MS/MS. Ser-262 in ICL3 was identified as a phosphorylation site on OX1R and the phosphorylated site is highlighted in red.

### 3.2 Expression and mutation of OX1Rs

To evaluate the functional importance of phosphorylation sites and exclude the

possibility of incorrect OX1R localization, mutated OX1R expression plasmids (OX1R-S262A and OX1R-S390A) were first constructed by site-directed mutagenesis of Ser to Ala, and then the cell-surface expression level of WT-OX1R, OX1R-S262A, and OX1R-S390A in HEK293T cells were investigated by TIRFM, ELISA and BRET. HEK293T cells were transfected with WT-OX1R-EGFP, OX1R-S262A-EGFP, OX1R-S390A-EGFP, and Golgi-EGFP and observed by TIRFM (488 nm); green spots were detected and visualized in cells expressing all OX1R-GFP constructs but not Golgi-EGFP (Fig. 2A). In addition, ELISA and BRET revealed no significant differences between WT-OX1R, OX1R-S262A, and OX1R-S390A (Fig. 2B and C). Three results suggested that compared with WT-OX1R, OX1R mutants had correct membrane location and no obvious effect on cell-surface expression of receptor, indicating that OX1R mutants were successfully constructed and trafficked to cell surface, which is a prerequisite for BRET.



**Fig 2. Cell-surface expression of WT-OX1R, OX1R-S262A, and OX1R-S390A**

(A) Visualization of EGFP-tagged WT-OX1R and mutant OX1Rs on the surface of living cells with TIRFM (green particles). Scale bars, 25  $\mu$ m. (B) Cell surface expression of WT-OX1R and mutant OX1Rs, quantified by ELISA. Results are expressed as a percentage of cell-surface expression levels of WT-OX1R, after correction for nonspecific expression in cells transfected with the empty vector. (C) Cell-surface expression of WT-OX1R and mutant OX1Rs, detected by BRET via the cell surface marker Kras-Venus and WT-OX1R-Rluc and mutant OX1Rs-Rluc. For ELISA and BRET, four independent experiments were performed with triplicate samples, and the results are expressed as means $\pm$ SEM of four experiments. ns, no significant difference, WT-OX1R vs OX1R-S262A and OX1R-S390A.

### 3.3 Ser-262 of OX1R is required for orexin A-induced receptor internalization

#### 3.3.1 Ser-262 Mutation of OX1R impairs its interaction with GRK2, but not with GRK5

GRK2 phosphorylates specific serines and threonines in the C-terminus of GPCRs, which leads to the desensitization and internalization of receptors upon  $\beta$ -arrestin recruitment. GRK2/5 is localized to the membrane, where it phosphorylates agonist-activated GPCRs, which then bind intracellular signaling partners via  $\beta$ -arrestin, leading to  $\beta$ -arrestin dependent signaling. Using BRET, we investigated the effects of Ser-390 in the C-terminus and Ser-262 in ICL3 on the interaction between OX1R and GRK2/5. The BRET assay provides real-time and dynamic insights, enabling analyzing protein-protein interactions in living cells. We determined both dose- and time-dependent effects of orexin A on the interaction. The dose- and time-response curves of GRK2 recruitment to OX1R revealed that the increase of BRET signals was dependent on orexin A concentration ( $10^{-10}$ – $10^{-5}$  M) in cells expressing GRK2-Rluc and WT-OX1R-Venus or OX1R-S390A-Venus. However, the mutant OX1R-S262A yielded a significantly lower BRET signal (Fig. 3A). Moreover, we observed that BRET signal increased rapidly after  $10^{-6}$  M orexin A treatment in cells expressing GRK2-Rluc and WT-OX1R-Venus or OX1R-S390A-Venus, and peaked at 10 min. Again, the mutant OX1R-S262A yielded a significantly lower BRET signal, which was similar to the negative control (GRK2-Rluc+ Venus) (Fig. 3B). These results indicate that the efficiency and affinity of OX1R-S262A raised by GRK2 was much lower than that of WT-OX1R and OX1R-S390A. In addition, we confirmed interaction between OX1R and GRK2 by cellular Co-IP. The interaction could be detected following  $10^{-6}$  M orexin A treatment after transfecting with both Myc-GRK2 and HA-OX1R, but not either plasmid alone. There was no OX1R and GRK2 interaction when expressing both Myc-GRK2 and OX1R-S262A (Fig. 3C).

By contrast, the dose- and time-response curves of GRK5 recruitment to OX1R revealed that BRET signals increased with orexin A concentration ( $10^{-10}$ – $10^{-5}$  M) in cells expressing GRK5-Rluc and WT-OX1R-Venus, OX1R-S262A-Venus, or OX1R-S390A-Venus (Fig. 3D). In addition, BRET signal rapidly increased after



stimulation with  $10^{-6}$  M orexin A in cells expressing GRK5-Rluc and WT-OX1R-Venus, OX1R-S262A-Venus, or OX1R-S390A-Venus, which peaked at 10 min (Fig. 3E). Critically, the OX1R-Ser-262 to Ala mutation blocked the interaction between OX1R and GRK2, indicating that Ser-262 of OX1R was required for orexin A-induced recruitment of GRK2 to activated receptors.

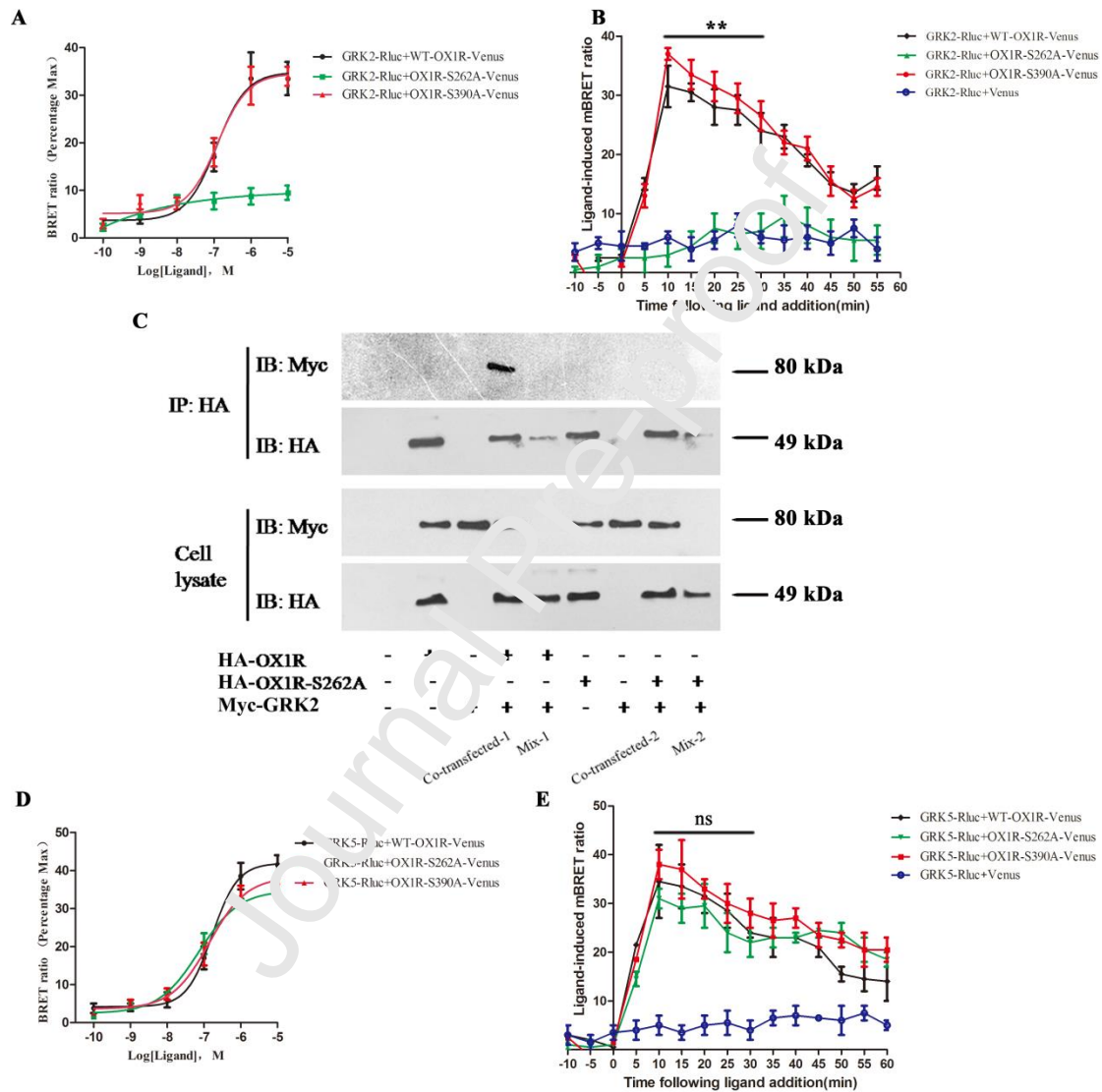


Fig 3. Ser-262 mutation of OX1R impairs its interaction with GRK2

HEK293T cells transiently transfected with Venus-tagged WT-OX1R or mutants-OX1R and GRK2-Rluc measured by BRET. (A) Dose-response curve of the recruitment of GRK2 to WT-OX1R and mutant OX1Rs, measured by BRET. After stimulation with different concentrations of orexin A, BRET signals were recorded between WT-OX1R or mutant OX1Rs and GRK2 at 15 minutes. (B) Time-dependent

curve of the recruitment of GRK2 to WT-OX1R and mutant OX1Rs, measured by BRET. BRET signals between receptor and GRK2 were recorded every 5 min before and after  $10^{-6}$  M orexin-A stimulation. Four independent experiments were performed with triplicate samples, and the results were expressed as means $\pm$ SEM of four experiments. **\*\*P<0.01 GRK2-Rluc+WT-OX1R-Venus vs GRK2-Rluc+OX1R-S262A-Venus.** (C) Interactions between GRK2 and WT-OX1R or mutant OX1Rs, measured by Co-IP. HEK293T cells were transfected with both HA-OX1R and Myc-GRK2 (co-transfected 1) or with either plasmid alone. The same cells were transfected with HA-OX1R-S262A and Myc-GRK2 (co-transfected 2) or with either plasmid alone. Samples containing either HA-OX1R or Myc-GRK2 were mixed (Mix 1), as were samples containing either HA-OX1R-S262A or Myc-GRK2 mixed (Mix 2). The results shown are representative images from three independent experiments. (D) Dose-response curve of the recruitment of GRK5 to WT-OX1R and mutant OX1Rs, measured by BRET. It is the same as the method of measuring GRK2. (E) Time-dependent curve of the recruitment of GRK5 to WT-OX1R and mutant OX1Rs, measured by BRET. Four independent experiments were performed with triplicate samples, and the results were expressed as means $\pm$ SEM of four experiments. ns, GRK5-Rluc+ WT-OX1R-Venus vs GRK5-Rluc+ OX1R-S262A-Venus.

### 3.3.2 Ser-262 mutation on OX1R does not affect its interaction with $\beta$ -arrestin1/2

To further study OX1R internalization, we used BRET to monitor the effects of C-terminal Ser-390 and ICL3 Ser-262 on the interaction between OX1R and  $\beta$ -arrestin1/2. The BRET signal following  $10^{-6}$  M orexin A stimulation was detected in HEK293T cells expressing  $\beta$ -arrestin1-Rluc and OX1R-S262A-EGFP.  $\beta$ -arrestin 1 was recruited to OX1R-S262A and OX1R-S390A similarly to WT-OX1R. We also observed a similar BRET signal after  $10^{-6}$  M orexin A stimulation in HEK293T cells expressing  $\beta$ -arrestin2-Rluc and OX1R-S262A-EGFP or WT-OX1R. These results indicating that OX1R Ser-262 is important for  $\beta$ -arrestin1/2 binding to OX1R does not affect the binding of  $\beta$ -arrestin1/2 and OX1R (Fig. 4, A and B).

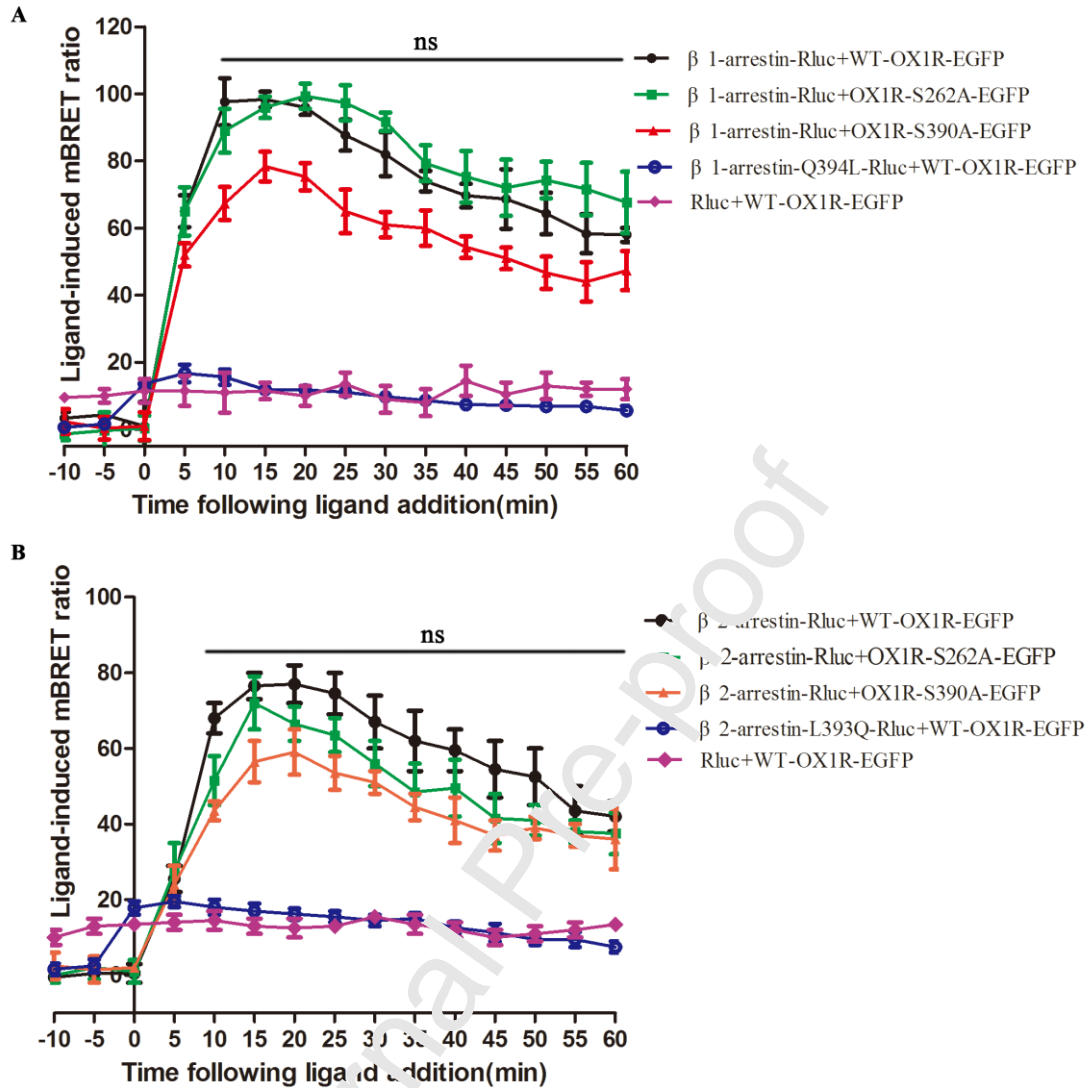


Fig 4. Ser-262 mutation of OX1R does not alter its interaction with  $\beta$ -arrestin1/2

Time-dependent curve of recruitment of  $\beta$ -arrestin 1/2 to WT-OX1R and mutant OX1Rs, measured by BRET. HEK293T cells transiently transfected with EGFP-tagged receptors and  $\beta$ -arrestin1-Rluc (A) or EGFP-tagged OX1R and  $\beta$ -arrestin2-Rluc (B), were treated with  $10^{-6}$  M orexin A. BRET signals between receptor and  $\beta$ -arrestin1/2 were recorded every 5 min before and after  $10^{-6}$  M orexin A stimulation. Four independent experiments were performed with triplicate samples, and the results are expressed as means $\pm$ SEM of four experiments. ns,  $\beta$ -arrestin1/2-Rluc+ WT-OX1R-EGFP vs  $\beta$ -arrestin1/2-Rluc+ OX1R-S262A-EGFP.

### 3.3.3 Ser-262 mutation of OX1R impairs its interaction with AP2

To detect the effect of OX1R Ser-262 on desensitization and internalization of

OX1R signaling, HEK293T cells co-expressing WT-OX1R-Venus or OX1R-S262A-Venus (shown in green) and mCherry-AP2 (shown in red) were stimulated with  $10^{-6}$  M orexin for various time intervals (0, 15, 30, and 60 min), and the cells were observed under confocal microscope. The results revealed that at 0 min (before agonist stimulation), there was clear cell surface distribution of OX1R (shown in green) and cytosol distribution of mCherry-AP2 (shown in red). After orexin A stimulation, there was internalization in HEK293T cells expressing WT-OX1R at 15 min. The internalization peaked at 30 min and then, at 60 min, cell surface distribution of OX1R was observed. There was co-localization of OX1R-Venus (shown in green) and mCherry-AP2 (shown in red) showing as moderate yellow fluorescence (Fig. 5A). By contrast, although we confirmed that the receptor was expressed, there was no obvious shift in OX1R-S262A-Venus localization and no co-localization with mCherry-AP2 after  $10^{-6}$  M orexin A stimulation for 60 min (Fig. 5B). ~~The results suggest that mutation of Ser 262 to Ala in OX1R can block OX1R internalization and endocytosis induced by orexin A.~~ The results showed that the mutation of Ser-262 in OX1R to Ala could reduce the internalization and endocytosis of OX1R induced by orexin A.

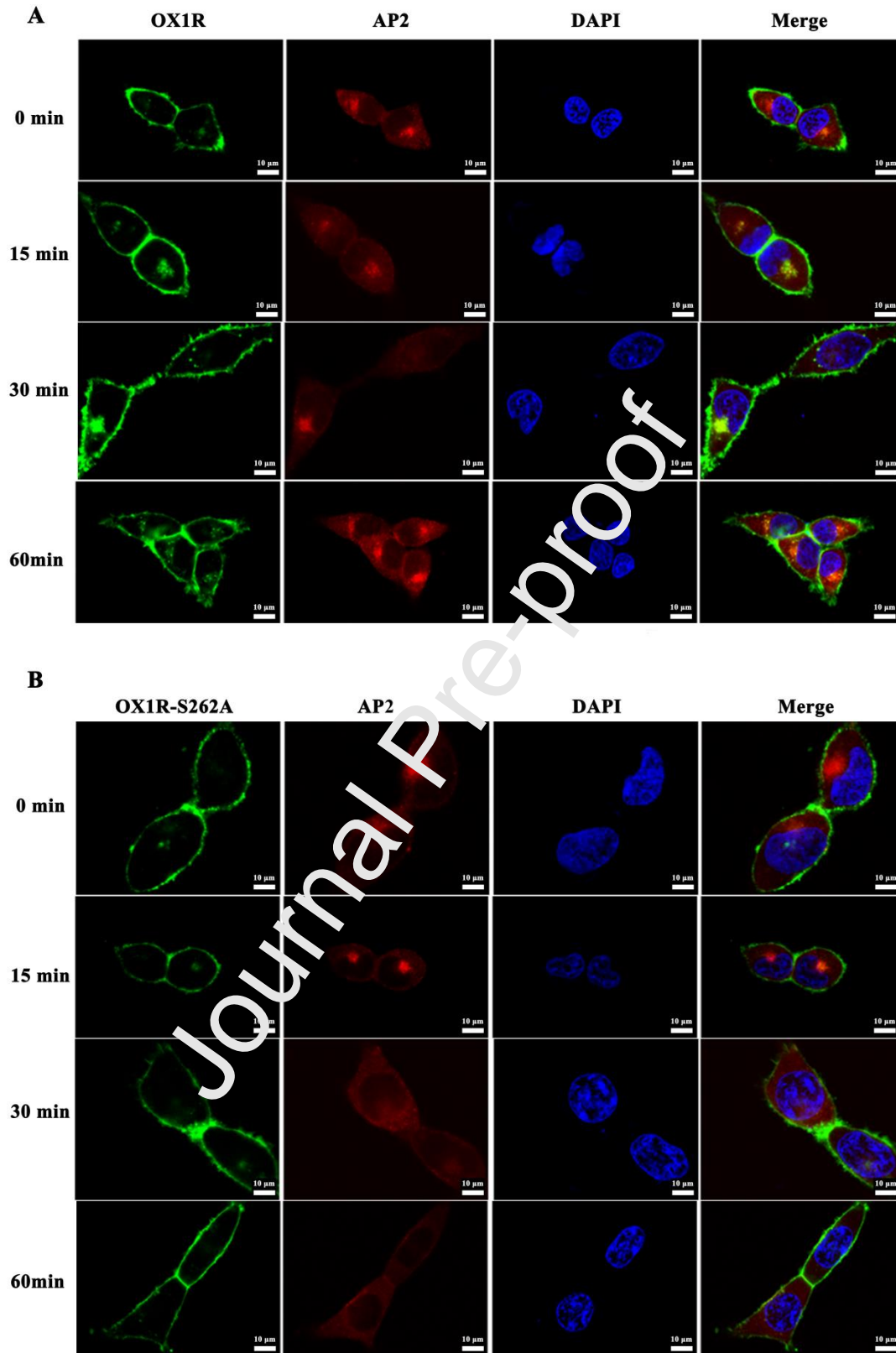


Fig 5. OX1R-S262A mutation impairs agonist-induced receptor internalization and endocytosis in HEK293T cells

HEK293T cells were co-transfected with WT-OX1R-Venus (A) or

OX1R-S262A-Venus (B) and mCherry-AP2, and cells were treated with  $10^{-6}$  M orexin A for various time intervals (0, 15, 30, and 60 min). Redistribution and co-localization (yellow) of receptors (green) and mCherry-AP2 (red) after orexin A stimulation are shown. Nuclei were stained with DAPI (blue). The results shown are representative images from at least three independent experiments. Scale bars, 10  $\mu$ m.

### 3.4 Ser-262 of OX1R does not alter its interaction with G Proteins

Upon agonist stimulation, OX1R can be coupled to  $G\alpha_q$  and  $G\alpha_{12}$ [25].  $G\alpha_q$  activation leads to an increase in the levels of its second messenger (intracellular calcium concentration) and its downstream effector, NFAT, whereas  $G\alpha_{12}$  activation leads to an increase in the level of the downstream effector SRF (Fig. 6C). First, we dissected the effects of Ser-262 of OX1R on  $G\alpha_q$ -dependent signaling by measuring intracellular calcium ions ( $Ca^{2+}$ ) using a Tristar LE 941 plate reader, and measuring NFAT using a double luciferase reporter assay. After stimulation by orexin A, changes of  $Ca^{2+}$  were detected in cells expressing WT-OX1R, OX1R-S262A, or OX1R-S390A. Dose-response curves were generated by nonlinear regression with a wide range concentrations of orexin A ( $10^{-11}$ – $10^{-5}$  M), and the half-maximal effective concentration (EC50) values was  $10^{-6}$  M (Fig. 6A). We then monitored changes in the ratio over time in the presence of  $10^{-6}$  M orexin A; the results revealed that treatment of WT-OX1R cells significantly increased the fluorescence signal. A similar effect was also noted in OX1R-S262A/OX1R-S390A cells (Fig. 6B). These results suggested that OX1R-Ser 262 did not alter intracellular  $Ca^{2+}$  upon agonist stimulation, further confirming that Ser 262 cannot affect the activation of  $G\alpha_q$ .

We also evaluated changes of NFAT in cells after stimulation with  $10^{-6}$  M orexin A. Relative to control cells (Mock), the activity of NFAT-luc was significantly higher and similar in WT-OX1R, OX1R-S262A and OX1R-S390A cells (Fig. 6D). These results imply that Ser-262 cannot alter NFAT activity, which involves the  $G\alpha_q$  pathway. We further dissected the effects of Ser-262 of OX1R on  $G\alpha_{12}$ -dependent signaling by measuring SRF with double luciferase reporter assay. Compared to mock control, the activity of SRF-luc was significantly lower and similar in WT-OX1R, OX1R-S262A and OX1R-S390A cells (Fig. 6E). These results suggest that Ser-262 cannot alter SRF activity, which involves the  $G\alpha_{12}$  pathway. Collectively, these findings show that OX1R-Ser-262 had no effect on G protein-dependent signaling.



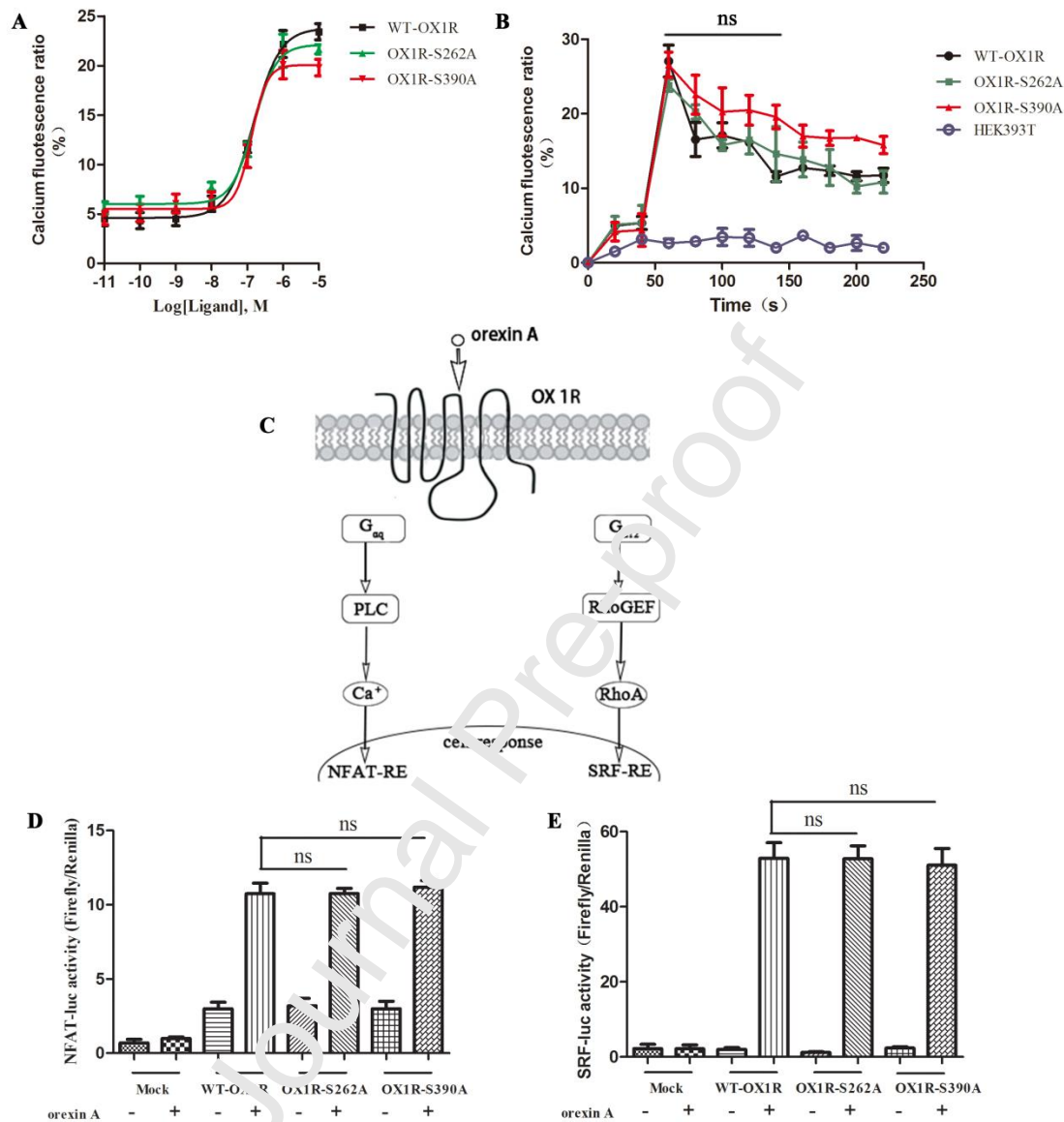


Fig 6. Ser-262 mutation of OX1R does not alter its interaction with G Proteins

(A) HEK293T cells transiently transfected with WT-OX1R or mutant OX1Rs, were treated with orexin A. Dose-response curves of intracellular  $\text{Ca}^{2+}$  for WT-OX1R and mutant OX1Rs at 30 seconds, measured by Fluo-4NM Calcium Assay Kit. Intracellular  $\text{Ca}^{2+}$  was measured using a Tristar LB941 plate reader. (B) Time-dependent curves of intracellular  $\text{Ca}^{2+}$  for WT-OX1R and mutant OX1Rs, measured by Fluo-4NM Calcium Assay Kit. Variations in intracellular calcium fluorescence over 230 s with  $10^{-6}$  M orexin A, assessed with a Tristar LB941 plate

reader. Four independent experiments were performed with triplicate samples, and the results were expressed as the means $\pm$ SEM of four experiments. ns, WT-OX1R vs OX1R-S262A. (C) Schematic of G protein-dependent signaling by OX1R. (D) and (E) Effects of WT-OX1R or mutant OX1Rs on NFAT and SRF activities. WT-OX1R, OX1R-S262A, OX1R-S390A respectively, twenty-four hours after co-transfection with NFAT-RE (D) or SRF-RE (E), the cells were treated with 1000 nM orexin-A 6 hours before harvest. NFAT and SRF activities were assayed using a Dual-Luciferase Reporter Assay System, and ratios of firefly to Renilla luciferase luminescence were calculated. Four independent experiments were performed with triplicate samples, and the results were expressed as the means $\pm$ SEM of four experiments. ns, WT-OX1R vs OX1R-S262A and OX1R-S390A.

#### 4. Discussion

GPCRs are the largest class of therapeutic targets in medicine, accounting for about a third of marketed drugs [26, 27]. GPCRs interact with a multitude of intracellular signaling pathways mediated by G proteins, and desensitization of these pathways is mediated by  $\beta$ -arrestins [28]. Signaling of most GPCRs via G proteins is terminated by the phosphorylation of active receptors by specific kinases (GRKs) and subsequent binding of  $\beta$ -arrestins, which selectively recognize active phosphorylated receptors. The interactions between GPCRs and  $\beta$ -arrestins determine the selectivity and efficiency of receptor signaling [29, 30]. Our preliminary studies showed that most GPCRs can be phosphorylated, and that phosphorylation of GPCRs occurs mainly in the C-terminal region or ICL3. Phosphorylation is one of the most important ways to regulate the function of GPCRs. Until recently, however, little was known about the phosphorylated sites of OX1R. In our study, first, putative OX1R phosphorylation site Ser-390 in the C-terminal region and Ser-262 in ICL3 were identified using NetPhos 2.0 prediction software. Second, we identified the key phosphorylated sites (Ser-262) on OX1R by LC-MS/MS in HEK293T.

GPCRs are generally of low natural abundance and overexpression is usually a prerequisite to their structural or functional characterization. The heterologous expression system provides the study of receptor function in well controlled cell environment, and also provides an alternative method for receptor function research in



different cell background. In our study, we used the recombinant cell lines, HEK293T, which is widely used cell lines for heterologous expression and the most used cells in orexin receptor studies to date. These cells have the advantage of expressing only a single orexin receptor subtype, which allows analysis of the receptor subtype differences. However, the cell background is very different from the native cells and the results may thus not be directly physiologically applicable.

$\beta$ -arrestin inhibit G protein activation and mediate GPCR internalization, and may also stimulate  $\beta$ -arrestin signal pathway. OX1R can bind orexin A (also known as hypocretin 1) with high affinity, but has considerably less affinity for orexin B (also known as hypocretin 2) [31]. Evans et al. have found human OX1R activation attracts  $\beta$ -arrestin 1/2 to the membrane in CHO cells, and moves into intracellular puncta with the continued presence of orexin A[32]. In our studies, we used orexin A to activate OX1R, and examined the roles of OX1R Ser-262 in G-protein and  $\beta$ -arrestin-dependent signaling in HEK293T with FRET and confocal microscope. We found that OX1R Ser-262 mutation weakened its interaction with GRK2, so that OX1R-S262A loses its ability to bind to GRK2, but did not alter its interaction with GRK5,  $\beta$ -arrestin1/2, or G-proteins, indicating that after Ser-262 was replaced with Ala, orexin A fails to induce GRK2 recruitment and terminates GRK2-dependent signaling (Fig. 7). For the G-protein dependent signaling, OX1R can be coupled to  $G\alpha_q$  and  $G\alpha_{12}$  in the presence of orexin A[33]. In our study, we found that Ser-262 cannot alter intracellular  $Ca^{2+}$  and SRF activity, indicating Ser-262 had no effect on G protein-dependent signaling.

AP2 is involved in clathrin-dependent endocytosis in which cargo proteins are incorporated into vesicles surrounded by clathrin (clathrin-coated vesicles, CCVs) which are destined for fusion with the early endosome[34]. The interaction between AP2 and OX1R was observed with a confocal microscope. OX1R-S262A-Venus and mCherry-AP2 were co-transfected into HEK293T cells. It was found that OX1R-S262A receptors were evenly distributed cell membrane surface (green) without orexin A stimulation (0 min). Then after different orexin A action time, it was found that the green fluorescence did not transfer to the cytoplasm, so it could not co-localize with the cytoplasm AP2. Therefore, through the above experiments, it is concluded that Ser-262 is the key phosphorylation site of OX1R endocytosis, and endocytosis will not occur after mutation. Taken together with our results, the mutation at Ser-262 of OX1R significantly disrupted GRK2 and AP2 depend signal,

but had little effect on the activation of G protein coupled with OX1R, and did not change the recruitment of GRK5 and  $\beta$ -arrestin.

Recently, intense efforts have been made to find signals that can be independently activated by agonist-induced receptor conformations or biased ligands, expanding the potential for new drugs aimed at these classic targets [35, 36]. Over the past decade, biased signaling, in which the activated receptor selectively engages only a subset of its potential intracellular partners, has attracted much attention in the GPCR field.  $\beta$ -arrestin bias sometimes confers positive effects, whereas G protein bias can cause adverse side effects [37, 38]. In this study, we identified a novel regulatory phosphorylation site (Ser-262) on OX1R that abolished its capability to interact with GRK2 and AP2, but did not affect its interaction with G proteins, GRK5, or  $\beta$ -arrestin1/2 activation. These results indicate the existence of biased signal transduction, which will benefit future drug development. The biased signal transduction may lead to the development of drugs with greater therapeutic potential and benefit, and fewer side effects, than current GPCR-targeted drugs for the prevention and treatment of insomnia, narcolepsy, and substance abuse. Similar conclusions were also arrived at in other studies, for example, G protein-biased ligand induces selectively GRK2-dependent signaling [39].

It seems that biased signaling could target specific physiological processes. However, whether this specificity is mediated by G-protein,  $\beta$ -arrestin, or both pathways is unclear, and the clinical ramifications are far from trivial. In the future, it will be necessary to investigate the relationship between physiological events and specific pathways. In addition, *in vivo* experiments are needed to further study that Ser-262 on OX1R regulates orexin A-induced GRK2 bias signal transduction, which is conducive to the development of new drugs.

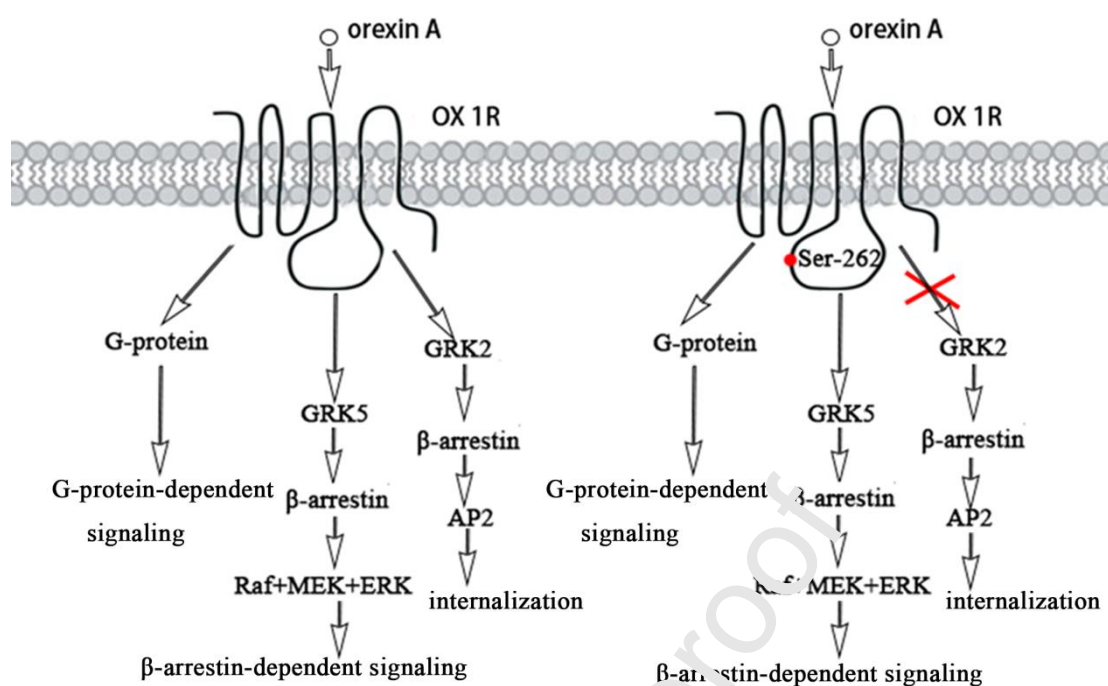


Fig 7. Schematic diagram of G protein-dependent or  $\beta$ -arrestin-dependent signaling of WT-OX1R and mutant OX1Rs

(A) Orexin A binds OX1R and triggers intracellular signaling events, including G protein-dependent and  $\beta$ -arrestin-dependent signaling pathways. (B) Orexin A fails to elicit GRK2 recruitment and terminates GRK2-dependent signaling when Ser-262 of OX1R is replaced with Ala.

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### Author contributions

Huannan Wang, Zhen Zhang, Rumin Zhang and Chunmei Wang performed the experiments; Dexiu Wang, Ruotong Wei, Ruotong Wei and Xiang Gao analyzed the data; Xin Cai wrote the manuscript; Jing Chen designed the experiments, wrote and revised the manuscript. All authors reviewed the results and approved the final

version of the manuscript.

### Conflicts of interest

The authors confirm that there are no conflicts of interest.

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#### Author contributions

Huannan Wang, Zhen Zhang, Rumin Zhang and Chunmei Wang performed the experiments; Dexiu Wang, Ruotong Wei, Ruotong Wei and Xiang Gao analyzed the data; Xin Cai wrote the manuscript; Jing Chen designed the experiments, wrote and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

## Highlights

A novel regulatory phosphorylation site (Ser-262) on OX1R

OX1R-Ser-262A that abolished its capability to interact with GRK2

No affect its interaction with G proteins, GRK5, or  $\beta$ -arrestin1/2 activation

Ser-262 is a key amino acid for OX1R internalization that contributes to induction of GRK2-dependent biased signaling

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